Nucleotide variations in the human genome

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Summary and general discussion

8.1 SUMMARY AND GENERAL DISCUSSION

Since the discovery of Nuclein by Friedrich Miescher during the winter of 1868/1869 [1], later named DNA [2, 3], great scientific progress has been made in the fields of biotechnology and genetics. The development of DNA sequencing and the polymerase chain reaction (PCR) accelerated developments leading to a new era in science and medicine [4, 5]. Using these techniques scientists were able to study specific DNA regions of interest, leading to the identification of variations in oncogenes and tumor suppressor genes (TSGs). Upon completion of the Human Genome Project (HGP), data on the complete human genome, including genomic diversity, became available [6-8]. The most common sequence variations found were Single Nucleotide Polymorphisms (SNPs), occurring approximately once every 100-1000 base pairs [9, 10] and are defined as a difference of a single base occurring at a particular genomic location [11]. Additionally, the term SNP is often used limited to single base variations with a frequency of occurrence of at least 1% in a particular population and to those that are non-disease conferring. Variations that do not meet these criteria are considered to be mutations rather than polymorphisms. Both restrictions however pose a dilemma. Firstly, a SNP in one population should be considered a mutation in the other due to population dependent occurrence of >1% vs. <1%. Secondly, a disease causing mutation of a single nucleotide in one individual could hypothetically be non-disease conferring in another and therefore should be considered a SNP. To overcome these issues the term SNP is used throughout this thesis as a single base variation on a certain genomic position regardless of occurrence and disease causing potential, that persists in a population.

Each individual has a unique set of these SNPs, ones SNP genotype can therefore be considered as a genetic fingerprint. SNPs occur less frequent in coding regions when compared to non-coding regions. Obviously, when located in a coding region a SNP may have a direct effect on the encoded protein [11-13]. SNPs in non-coding regions however, may still have a functional effect due to altered expression of the protein involved [14-16]. Because of the large amount of SNPs throughout the entire human genome and their possible effect on encoded proteins and gene expression, variations in SNPs do attribute largely to human phenotypic diversity. Because of their presence throughout the genome and frequent occurrence, SNPs are hypothesized to be highly useful markers with regard to tissue identification and loss of heterozygosity (LOH) and allelic instability testing of cancer critical stretches of DNA.

8.1.1. Technological advances

Due to several technological advances during the last decade(s) the use of routine molecular techniques has increased greatly. Currently, (real time) PCR is often the technique of choice of many routine molecular laboratories for a diverse range of diagnostic applications. At

present, routine tests include for example the detection of bacteria and viruses [17-23], neoplasm-associated mutations [24-26] and up- and down-regulation of mRNA's [27]. The (real time) PCR technique is therefore widespread and many laboratories are skilled users. However, more "specialized" molecular techniques are currently used for various purposes such as tissue identification with regard to sample mix-ups, LOH testing of clinically relevant genomic loci in neoplasms and allelic imbalance of cancer critical genes. Real time PCR is an excellent and relatively easy tool to detect SNPs and it may therefore be particularly useful when detecting SNPs with regard to these applications.

CHAPTER 2 | OPTIMIZING DNA EXTRACTION

In several studies described in this thesis, extraction of DNA from formalin-fixed paraffin-embedded (FFPE) tissues is an important primary process on which the reliability of the downstream analysis partly depends. DNA extraction from FFPE tissues suffers from difficulties due to formalin-fixation induced cross-links between tissue proteins and DNA, which may result in blocking of amplification. Secondly, DNA may be fragmented due to aging of the specimen or the pH of the fixative [28].

In chapter 2, a comparative analysis of four DNA extraction methods is described for extraction of DNA from FFPE tissues. The methods tested were heat-treatment, QIAamp, EasyMAG and Gentra. All methods were tested with and without proteïnase K digestion as a pretreatment. The resulting DNA extracts were tested regarding i) the cumulative effect of extraction efficiency and PCR inhibition by monitoring amplification of an internal control DNA virus, ii) the performance in SNP analysis by real time PCR and iii) performance in a conventional multiplex PCR amplifying 200, 400 and 600 bp human DNA fragments. Gentra DNA extraction was found not suitable for purifying DNA from FFPE samples in general. This was possibly due to the method's inability to remove protein-DNA cross-links and insufficient lysis, both resulting in the loss of DNA during washing steps. Proteïnase K digestion was found to be indispensable for optimal DNA purification from FFPE tissues, as was previously described [29, 30]. Although the heat-treatment, QIAamp and EasyMAG methods were all successful in extracting DNA, the latter two methods performed best for real time SNP detection. Amplification of the 400 and 600 bp amplicons was found to be most successful using QIAamp extracts followed by the use of extracts from heat-treatment and EasyMAG. Hands-on-time was lowest using heat-treatment followed by EasyMAG and QIAamp (when extracting 24 samples: approximately 60 min. for QIAamp versus 5 min. for heat-treatment and 25 min. for EasyMAG).

We concluded that the extraction methodology strongly influences the downstream molecular analyses, which is in line with previous findings [28, 31-35]. Therefore, the choice of which extraction is best to be employed should be based on the envisioned downstream

molecular analyses. For the experiments described in this thesis, when extracting DNA from FFPE tissues in combination with real time PCR based detection of SNPs, the EasyMAG extraction method was found most suitable.

CHAPTER 3 AND 4 | CONFIRMING THE IDENTITY OF HUMAN TISSUES

Although specimen identification is a carefully controlled factor during pre-laboratory handling in clinical laboratories, mislabeling does occur. Unfortunately, the patients involved may suffer from severe consequences due to these sample mix-ups. Therefore issues of potential sample mix-up need to be addressed. When focusing on surgical pathology and its main sample type, FFPE tissue, error rates are reported ranging from 0.08 to 1.2% [36-41]. A second widely used patient material for diagnostic and treatment related purposes is serum. The percentage of mislabeling when handling serum is estimated to be around 0.1% [42-45]. These error rates further emphasize the need for thorough identification of clinical samples to address suspected mix-ups. Numerous techniques for the detection of specimen mix-ups have been described. Most rely on characterization of DNA repeats [46-48], human leukocyte antigen system (HLA) [49-54], or other polymorphic genetic loci [50]. In addition, commercial identification kits are available [55-57]. However, implementation of these methods is hampered by several issues such as the need for expensive sequencing equipment, contamination risk caused by post-PCR processing, poor DNA quality (FFPE tissues) and low DNA quantity (serum). To overcome these issues, a SNP profiling assay suitable for identity confirmation of FFPE tissues and serum samples was developed and described in chapter 3 and 4. The assays applicability was demonstrated by means of several cases of potential mix-ups.

To maximize the chance that an individual could be distinguished based on their SNP profile while analyzing a limited number of SNPs, a symmetrical distribution of alleles (minor and major allele frequencies of approximately 0.5) was chosen. A panel of 17 SNP assays, targeting SNPs distributed over 16 different chromosomes, was selected. Three SNPs were excluded from the final recommended panel of SNPs due to a significantly (chi-square test; *P* value of <0.05) differing allele frequency within the population tested. Four SNPs (2 of which were also excluded due to deviant allele frequencies) were excluded based on an *in silico* investigation of all 17 SNPs on whether the SNP loci were located in frequent LOH regions. Finally, 2 SNPs were excluded based on their technical limitations. Employing a final panel of 10 SNPs with an approximate MAF of 0.5 and assuming their genotype frequencies to be in the Hardy-Weinberg equilibrium [58, 59], the chance for 2 randomly chosen individuals to have the same SNP profile is 1 in 18,000. All described cases of potential sample mix-up (total n=7; FFPE tissues n=6; serum samples n=1) were resolved and our findings were confirmed by additional histological and serological testing, respectively.

The currently available assays have an extraordinary high average probability of identity of up to 5 x 10^{12}. Evidently, such high average probabilities of identity are needed in court. For use in cases of potential mix-ups however, the accuracy of our test (1 in 18,000 average probability of identity) appeared sufficient in all cases handled so far. If a higher accuracy is necessary, the average probability of identity generated by our SNP profiling assay could easily be enlarged by increasing the number of SNP assays included. When analyzing 20 or even 30 SNPs with approximate allele frequencies of 0.5, the discriminatory power of our test would rise to 3.3 x 10^{8} and 6 x 10^{12}, respectively. SNP profiling has the advantage that very small stretches of DNA (around 80 bp) are amplified in contrast to other techniques requiring up to 360 bp. Furthermore, SNP profiling can be performed on any standard real time PCR cycler and does not need expensive sequencing equipment. Other significant advantages of our real time PCR based procedure are its quick turnaround time and the lack of post-amplification handling, strongly reducing the risk of amplicon contamination.

CHAPTER 5 | DETERMINING *JAK2V617F* MUTATIONAL STATUS

The *Janus Kinase 2* (*JAK2*) gene encodes the JAK2 tyrosine kinase [60]. JAK2 plays a major role in mediating cytokine-signaling and therefore has a large impact on haematopoiesis [61]. A G-to-T missense mutation in *JAK2* results in a valine-to-phenylalanine (V-F) amino acid substitution at codon 617 (*JAK2V617F*) and subsequently constitutive activation of the JAK-STAT pathway [60, 62-66]. *JAK2V617F* is commonly found in myeloproliferative diseases (MPD), such as polycythemia vera (PV), essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (IMF) [62-65]. In addition to mutation detection, a correlation between *JAK2V617F* mutant allele burden and severity of disease has been postulated [67-76].
To assess the *JAK2V617F* mutational status in patients suspect of MPD a real time PCR suited for sensitive detection and quantification of this mutant allele amongst wild-type DNA was developed and described in chapter 5.
To maximize the sensitivity of our real time PCR, amplification of the wild-type allele was blocked. Two different blockers were tested, one was a locked nucleic acid (LNA) oligonucleotide the other a peptide nucleic acid (PNA) oligonucleotide. Sensitivity and linearity of both blocking strategies were compared by determining the limit of detection using twofold (v/v) serial dilutions (0.006% to >97% *JAK2V617F* DNA diluted in wild-type DNA) of genomic DNA from a patient with PV in genomic DNA of a *JAK2V617F* negative donor. The assay combined with the PNA oligonucleotide linearly detected *JAK2V617F* with a for quantification acceptable (mean Ct-value ± 1 Ct) reproducibility ranging from approximately 0.05% to 97%. This range was determined for the real time PCR with the LNA oligonucleotide to be 0.2% to 97%. The developed real time PCR assay in combination with the PNA oligonucleotide has an equal sensitivity to techniques such as allele-specific PCR/amplification refractory mutation

system, allelic discrimination, and PCR-MALDI-TOF. A similar real time PCR employing an LNA blocking oligonucleotide described in 2006, yielded a sensitivity of 0.01% [77, 78]. However, it's sensitivity was determined using a HEL cell line, which is known to carry more than two mutant alleles per cell, instead of patient DNA [79]. When analyzing a cohort of 100 healthy individuals (blood donors) of <30 years of age utilizing the real time PCR in combination with the PNA oligonucleotide a background signal was observed in 9% of the healthy donors. In one healthy donor a weak positive/background signal was observed in duplicate. Firstly, these background signals could be caused by technical limitations of the assay. Secondly, *JAK2V617F* could in fact be present in these patients in an extremely small amount of circulating cells. Although similar observations have been made with other *JAK2V617F* assays, its clinical significance remains unclear [78, 80, 81]. To prevent false positives in a clinical setting a cut-off Ct-value of ≥37.9 (based on the mean Ct-value of the negative cohort ± 3*SD) was determined.

The presented assay has been employed for diagnostic use for over a year. The generated results indicate a robust and rapid test with excellent reproducibility: the overall CV with different batches of controls, reagents, and various real time PCR cyclers was 1.5% for the 1% *JAK2V617F* control (n = 27) and 1.4% for the 50% *JAK2V617F* control (n = 24). Because reproducibility is essential for a reliable quantitative assay [82] these observations confirm that the developed assay is well suited for the intended application.

CHAPTER 6 | LOSS OF HETEROZYGOSITY OF THE *JAK2* GENOMIC REGION

Loss of heterozygosity (LOH) is a genetic incident where heterozygosity at a chromosomal locus within an organism's germline DNA is lost in a somatically altered (cancer) cell. LOH can therefore contribute to the accumulation of genetic alterations during carcinogenesis [83-85]. LOH may occur through several major mechanisms such as partial and whole chromosome loss [83, 86]. Another LOH causing mechanism is mitotic recombination leading to acquired uniparental disomy (aUPD) [62, 87]. When aUPD is preceded by an oncogene activating mutation, such as *JAK2V617F*, resulting in homozygosity of the mutant allele, the effect of the mutation may be further potentiated [62]. *JAK2V617F* mutant allele burdens in general and mutant allele burdens of >50% in PV and ET are correlated with severity of disease [67-72, 74-76]. In the case of aUPD, conventional cytogenetic analyses techniques are not able to detect such an abnormality [88]. Currently, STR typing and SNP oligonucleotide genomic microarrays are used to assess LOH status [88]. Both techniques however are less accessible to routine molecular diagnostic laboratories, hampering the implementation of these clinically relevant diagnostic tools.

A real time PCR based SNP assay was developed to detect LOH of the *JAK2* region and is described in chapter 6. The LOH test consisted of a panel of 10 real time SNP PCRs. Obviously,

when testing for LOH only heterozygous SNPs are informative. To maximize the chance that an observed SNP profile was informative only SNPs with an approximate MAF of 0.5 were included in the SNP panel. To determine which SNPs were informative, the germline SNP profile was determined for all included patients using archived, non-neoplasm related FFPE tissues.

The *JAK2V617F* detection and quantification assay described in chapter 5 was used to select a group of 12 blood samples containing the *JAK2V617F* mutation. Six of these harbored a mutant allele burden of 25-50% and the remaining 6 above 50%. This panel was used to test our *JAK2*LOH SNP assay. Subsequently, 81 patients suspect of a myeloproliferative neoplasm (MPN) were tested with the *JAK2*LOH assay and the *JAK2V617F* mutational status was determined. All 93 samples were simultaneously tested with regard to *JAK2*LOH status using an STR assay as previously used by Kralovics and coworkers in 2005 [62]. In the *JAK2V617F*-positive patient cohort 2 of the 5 patients with a mutant allele burden of 25-50% harbored *JAK2*LOH. This indicates that in addition to *JAK2V617F* detection and quantification, determining the *JAK2*LOH status is of value as well. These findings are in line with those of Scott and colleagues, describing that a large number of PV patients harbor homozygous subclones, which may predominate over time [89]. As expected all 6 patients with a mutant allele burden larger than 50% were found to have *JAK2*LOH. In the patients suspect of MPN cohort a total of 6 patients were found to have *JAK2*LOH. In none of the *JAK2V617F*-negative patients *JAK2*LOH was detected. Considering the STR assay to be the gold standard, our SNP assay had a sensitivity and negative predictive value of 100% for both the *JAK2V617F*-positive patient cohort and the patients suspect of MPN cohort. Positive predictive values of the SNP assay were 85.7% and 100% and the specificity was 80% and 100% in the respective cohorts. The SNP assay was found to have a higher success rate than the STR assay (3 vs. 9 non-informative profiles) in this population. Additionally, the SNP assay was found to provide more detailed information with regard to ROH/LOH status of the *JAK2* gene and surrounding loci.

CHAPTER 7 | ALLELIC IMBALANCE AT THE *HER2/TOP2A* LOCUS

Breast cancer is a heterogeneous disease displaying a diverse variety of biological features. The estrogen and progesterone receptors (ER, PR) are proteins well known to be expressed excessively by these tumors [90-92]. Categorizing breast cancers based on the level of expression of these proteins is utilized for the prediction of clinical outcome and therapy selection [93]. Available treatments in ER and PR positive breast cancer cases are e.g. Tamoxifen and aromatase inhibitors [90-92].

The *HER2* oncogene encodes the human epidermal growth factor receptor 2 protein [94, 95]. HER2 plays a pivotal role in the integrate network of HER signal transduction involving a multitude of pathways [96-98]. *HER2* amplification, found in approximately 25-30% of

breast cancers, results in HER2 overexpression and subsequent overstimulation of the pathways involved [96, 99]. Patients with *HER2* amplified breast tumors benefit from treatment with Trastuzamab (Herceptin), a monoclonal antibody that is thought to block HER2 mediated HER signal transduction [100, 101]. The juxtaposed *TOP2A* gene encoding toposiomerase II alpha coamplifies in 30-45% of *HER2* amplified cases [102-104]. TOP2A is an enzyme that catalyzes the topological DNA changes needed during cell division [105]. *TOP2A* amplification is a favorable characteristic in patients treated with anthracycline (AC)-containing adjuvant chemotherapy [102-104]. The opposite is true for *TOP2A* deletion, which is found in 15-40% of *HER2* amplified cases [102-104]. ACs are chemotherapeutic agents known to exert *TOP2A* inhibiting properties. It is these properties that are suggested to be the biological rationale behind the added benefit from adjuvant AC therapy in tumors with *TOP2A* amplification [105-107]. In fact, the association of improved AC response rates in *HER2* amplified tumors is now suggested to be attributed to *TOP2A* coamplification [104]. Tumors lacking ER, PR and HER2 overexpression are defined as "triple negative" (TN) breast tumors. TN tumors are associated with poor clinical outcome and lack of (currently) available targeted therapy [108, 109]. In addition to these well established biological characteristics, distinct gene-expression profiles and genetic alterations may yield added information suitable to subcategorize these breast cancer types [110-116]. Copy number alteration of the genomic region incorporating the *HER2/TOP2A* locus is suggested to be a marker for the degree of chromosomal instability in breast cancer [117, 118]. The higher degree of chromosomal instability has been associated with better response to adjuvant AC-based chemotherapy [114]. To be able to employ such *HER2/TOP2A* allelic instability as a possible adjuvant molecular marker in a diagnostic setting, a real time PCR based SNP assay was developed and described in chapter 6. Eleven SNPs on the *HER2/TOP2A* locus were selected. The SNPs were chosen based on their location within the smallest region of *HER2* amplification (SNP 1-6) [119] and up- and downstream of *TOP2A* (SNP 7-11). Additionally, SNPs were chosen with a MAF of 0.5 amongst different ethnic groups to maximize the informative value of the assay independent of patients' ethnicity. The germline SNP profile of each patient was determined by analyzing a histologically normal breast or lymph node FFPE tissue.

Fourty-four patients with 3 different entities of primary breast cancer were included: 1) a HER2 overexpressed (HER2+) group (n=15), 2) a triple negative (TN) group (n=16) and 3) a ER and PR positive (ERPR+) group (n=13). As a control group, histologically normal FFPE tissues of 10 patients with no breast tumor were included. As expected, all informative SNP results from the breast tissues in the control group fell within the range of normal data distribution. In the histologically normal samples from the 44 breast tumor patients, only 1 of 208 informative SNPs was found to be equivocal, indicating well established cut-off values for statistical allelic (im)balance calling. Amongst the HER2+ group, all patients (15/15) showed SNP results indicative of allelic imbalance. In 2 patients, a portion of SNP results were

indicative of allelic imbalance, while the remaining SNPs were normal. These observations were possibly caused by several small mutations each affecting part of the locus involved. Of the 16 triple negative tumors, 12 (75%) displayed instability. This is in line with previous findings where the complex genomic profiles, with numerous gains and losses, of these tumors were classified as "sawtooth patterns" [110-115]. Three (19%) samples from the TN group displayed no instability, and 1 was inconclusive. Of the 13 hormone receptor positive tumors, 5 (38%) displayed allelic imbalance, while 8 (62%) did not. Although the number of samples in our study is limited, the portion of tumor samples that contain *HER2/TOP2A* allelic instability in the TN and ERPR+ group (75% and 38%, respectively) roughly corresponded to the AC-containing adjuvant chemotherapy response rates found within these groups (85% and 47%, respectively) [120]. To establish whether the presented SNP test provides added diagnostic value, a clinical study should be performed in which ample size TN and ERPR+ patient groups are included with 5 year clinical follow-up data. Such a study could provide information with regard to allelic instability at the *HER2/TOP2A* locus as a marker for the prediction on clinical outcome and its possible use for therapeutic decision making.

8.2 VENTURING BEYOND THE SCOPE OF THIS THESIS: BACTERIAL SNP GENOTYPING

In addition to the focus on various human diagnostic applications of real time PCR based SNP profiling, we investigated its employability in bacterial genotyping.

During a 4-year period, from 2007 to 2010, The Netherlands was confronted with the largest *Coxiella burnetii* –the causative agent of Q-fever [121, 122] - epidemic ever, with approximately 3,500 notified human cases at the end of 2009 [123]. Since epidemiological data concerning this outbreak were lacking and no direct typing method was available at that time we set out to explore the possibilities of *C. burnetii* SNP genotyping. A panel of 10 discriminatory SNPs was firstly used to genotype an international panel of 28 reference strains, which were previously typed by Multi Locus Sequence Typing (MLST) [124, 125] and 5 commercial strains. Secondly, 40 outbreak related samples (14 of human origin and 26 of animal origin) were SNP genotyped. Thirdly, the developed SNP genotyping assay was employed to determine whether the mandatory vaccination of dairy goats with the formalin inactivated Coxevac® *C. burnetii* vaccine could result in excretion of *C. burnetii* (DNA) in the milk. Such false positive bulk milk results could lead to an unjustified culling of animals. Six adult dairy producing goats were subcutaneously inoculated twice with Coxevac® and 2 were not inoculated as negative controls.

The international panel revealed a total of 9 SNP genotypes, representing 14 MLVA types. The outbreak panel consisted of a broad spectrum of human and animal sample types on which SNP genotyping could directly be successfully employed. We were able to identify 5

distinct genotypes: 3 in human samples and 4 in samples derived from livestock. This implies that environmental circumstances (such as the high density of farms and people in the same area, dry periods during spring) rather than one highly virulent *C. burnetii* strain favored the Dutch Q fever spread. In the six vaccinated goats, *C. burnetii* DNA became detectable in milk samples from a few hours after the first vaccination until day 10. The 2 control goats remained negative throughout the experiment. SNP profiling showed an identical profile to that of the Coxevac® vaccine, which was not observed in any other outbreak related sample. This strongly indicates the vaccine being the source of the *C. burnetii* DNA in milk. Our observation had implications for the policy of the Dutch Ministry of Agriculture, Nature and Food Quality to combat the spread of the ongoing Q fever epidemic in the Netherlands. The strategy of simultaneous vaccination of dairy goats and bulk tank milk testing to identify contaminated farms was reconsidered and a 2-week interval between vaccination and bulk tank milk testing was imposed.
Our findings were published in *Applied and Environmental Microbiology* [126] and *Vaccine* [127] in 2011.

8.3 CONCLUDING REMARKS

As demonstrated by the studies described in this thesis, real time PCR based SNP profiling currently has a broad range of diagnostic applications. In combination with the relative ease of implementation of the technique in any molecular laboratory makes real time SNP profiling an attractive diagnostic tool. We therefore hypothesize that the presented assays form a starting point for the development of other clinically relevant diagnostic tests with regard to loss and/or gain of cancer critical stretches of DNA and possibly even interlaboratory bacterial genotyping. Additionally, broad implementation and use of the SNP assay to confirm the identity of all human clinical samples in a hospital setting would result in an improved securing of downstream evaluations and critical decisions concerning the patients involved. To realize this concept, the number of targeted SNPs should be increased to reach sufficient discriminatory strength. The costs could be reduced drastically by performing the corresponding real time PCRs in small reaction volumes (<5 µl) using 384- or even 1536-well platforms.
Ultimately, whole genome sequencing could make many currently employed molecular diagnostic assays redundant in the future, including real time PCR based SNP profiling. However, the corresponding equipment and techniques needed are very expensive and not accessible for most molecular laboratories. Additionally, analysis of the extremely large amounts of generated data is still difficult to handle and interpret. Real time PCR may therefore be the most probable technique of choice in most molecular laboratories, currently and in the near future.

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